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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Peptide Synthesis on Chitosan

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(57) 18 Claims

Notice: This application is as filed and may therefore contain an incomplete specification.



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**ABSTRACT**

Chitosan, a partially deacetylated derivative of chitin, is used as a support for peptide synthesis. The resultant peptide-chitosan complex can be used directly for injection into an animal. The support is biodegradable and exhibits little or no antigenic properties. The sequence of the resultant structure is specific and, as such, is predictable and controllable.

## PEPTIDE SYNTHESIS ON CHITOSAN

5

## FIELD OF THE INVENTION

10 The present invention relates to a support for peptide synthesis and peptide antigen amplification, and in particular, to a chitosan support therefor.

## BACKGROUND OF THE INVENTION

15 Synthetic peptides are currently used for the development of sequence specific antibodies and are being incorporated into highly specific vaccines. However, the peptides must generally be presented as part of larger molecules in order to function as effective immunogens and  
20 must be co-injected with an adjuvant in order to obtain an optimum response.

In order to present peptides as larger molecules, the peptides are often conjugated to a carrier or support. One such support is a carrier protein, such as keyhole  
25 limpet haemocyanin. However, the peptide is randomly coupled to the protein, usually through free amino groups, resulting in a non-specific peptide presentation and a structure which is difficult to predict and to control. Moreover, the carrier protein itself may be a multiepitope  
30 antigen. This can be a serious disadvantage, especially in vaccine development. In particular, the protein carrier could lead to an adverse response, for example, by generating an auto-immune response.

35 In an effort to overcome the problems associated with the use of protein carriers, researchers have synthesized peptides onto a branching lysine core resulting in a multiple antigenic peptide (MAP) structure (Posnett,

D.N. et al J Biol Chem 263: 4: 1719-1725; 1988). The MAP construct provides control over the resultant structure and peptide presentation. However, there are also disadvantages associated with the use of MAP. In particular, it was found that antibodies raised against the MAP did not always cross-react with the cognate protein (Briand, J.P. et al J Immunol Methods 156: 255-265; 1992).

Furthermore, the MAP is typically synthesized on a solid phase resin support, from which the complex must generally be cleaved following synthesis. Cleavage of the complex from the support, usually with anhydrous hydrofluoric acid, can cause side reactions. The reattachment of side chains results in a non-uniform complex. The side chains also represent impurities which may cause immunology problems.

Chitin is a naturally-occurring biodegradable polysaccharide that forms a base for the hard outer integuments of crustaceans, insects and other invertebrates. Chitosan is derived from chitin by deacetylation.

Chitosan has been used as an adsorbent for affinity chromatography (Moriguchi et al, United States Patent Number 4,879,340; November 7, 1989) and as a carrier for immobilized enzymes (Kawamura et al, United States Patent Number 4,833,237; May 23, 1989). Chitosan has also been used as a carrier for antigen or antibody (Unitika Kabushiki Kaisha, Japanese Patent Application Number 89012280-B; February 28, 1989). Prior to coupling the antigen or antibody, the chitosan is treated with a reagent having at least two functional groups reactive with the amino groups and the hydroxyl groups of the chitosan support. However, as discussed hereinabove with respect to carrier proteins such as keyhole limpet haemocyanin, the antigen or antibody is randomly coupled, resulting in a non-specific peptide presentation and a structure which is difficult to predict and to control.

An object of the present invention is to synthesize a peptide on a support having little or no antigenic

properties which can then be used directly for injection into an animal. The sequence of the resultant structure is specific and, as such, is predictable and controllable.

5

#### SUMMARY OF THE INVENTION

According to one aspect of the present invention, there is provided a process for peptide synthesis on chitosan, comprising the steps of: providing amino acids having side-chain reactive groups thereof blocked with blocking groups; protecting the  $\alpha$ -amino group of the amino acids with a temporary protecting group; attaching a first amino acid to the free amino groups of the chitosan; releasing the temporary protecting group from the attached amino group; sequentially attaching the desired amino acids to the previously attached amino acid and releasing the temporary protecting group therefrom until the desired peptide is synthesized to form a peptide-chitosan complex; and removing the blocking groups from the side-chain reactive groups of the amino acids.

According to another aspect of the present invention, there is provided a peptide-chitosan complex prepared by the process comprising the steps of: providing a chitosan support; providing amino acids having side-chain reactive groups thereof blocked with blocking groups; protecting the  $\alpha$ -amino group of the amino acids with a temporary protecting group; attaching a first amino acid to the free amino groups of the chitosan; releasing the temporary protecting group from the attached amino group; sequentially attaching the desired amino acids to the previously attached amino acid and releasing the temporary protecting group therefrom until the desired peptide is synthesized to form a peptide-chitosan complex; and removing the blocking groups from the side-chain reactive groups of the amino acids.

According to a further aspect of the present invention, there is provided a peptide prepared by the

process comprising the steps of: providing a chitosan support; providing amino acids having side-chain reactive groups thereof blocked with blocking groups; protecting the  $\alpha$ -amino group of the amino acids with a temporary protecting group; attaching a bifunctional cleavable linker molecule to the free amino groups of the chitosan; attaching a first amino acid to the free amino group of the cleavable linker molecule; releasing the temporary protecting group from the attached amino group; sequentially attaching the desired amino acids to the previously attached amino acid and releasing the temporary protecting group therefrom until the desired peptide is synthesized to form a peptide-chitosan complex; cleaving the cleavable linker molecule to release the peptide from the chitosan; and removing the blocking groups from the side-chain reactive groups of the amino acids.

#### BRIEF DESCRIPTION OF THE DRAWINGS

In drawings which illustrate embodiments of the present invention:

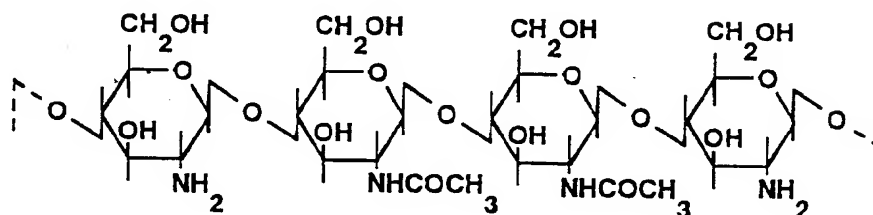
Figure 1 is a graphical representation of an elution pattern of the crude product of Example 1;

Figure 2 is a graphical representation of antibody response to an hPTH-(44-68)-chitosan complex prepared in accordance with the present invention; and

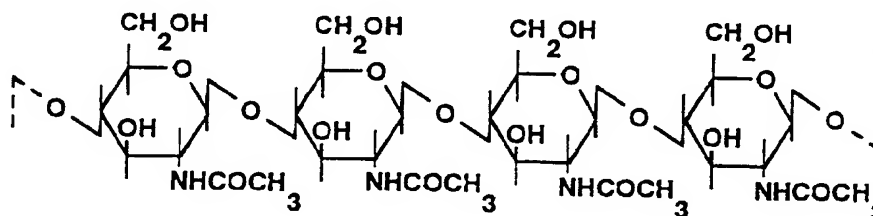
Figure 3 is a graphical representation of antibody response to an hPTH-(1-17)-chitosan complex prepared in accordance with the present invention.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, chitosan, having the following formula:



is used as a support for peptide synthesis. Chitosan is produced from chitin, having the following formula:



which is an N-acetylated polymer of 2-desoxy-2-amino glucose, and is substantially inert to the immune system. A portion of the residues of chitin are deacetylated in the production of chitosan, leaving some amino groups free as nuclei for peptide synthesis. The deacetylation reaction is performed in a manner known to those skilled in the art (for example, Neugebauer et al, Carbohyd Res 189: 363-367; 1989).

In accordance with the present invention, once the structure of a synthetic peptide is determined, solid phase peptide synthesis is conducted by a stepwise coupling of the desired amino acids to the chitosan. The technique of solid phase synthesis developed by R.B. Merrifield (Advances in Enzymology 32: 221-296; 1969) is widely and successfully used for the synthesis of polypeptides, such as parathyroid hormone. The strategy is based on having the carboxyl-terminus of the amino acid attached to a solid support. Successive amino acids are then added in a very high yield.

A first amino acid is prepared for solid phase peptide synthesis by blocking reactive side groups, for example the carboxyl group in glutamic and aspartic acids and the side-chain amino group in lysine, in a manner known to those skilled in the art. Suitable side-chain protecting groups are methoxytrimethylphenylsulfonyl (Mtr) for arginine; t-butyl for aspartic acid, glutamic acid and serine; trityl for asparagine, glutamine and histidine; and t-butyloxycarbonyl for lysine.

The  $\alpha$ -amino group of the amino acid is then protected by a fluorenylmethyloxycarbonyl (Fmoc) group and the carboxyl-terminus of the Fmoc-amino acid is activated for coupling to the free amino groups of the chitosan with, for example, a TBTU/HOBT/NMM (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/hydroxybenzotriazole/N-methylmorpholine) coupling procedure. The Fmoc group acts as a temporary protecting group on the  $\alpha$ -amino groups of the amino acid and can be removed under mild alkaline conditions without affecting the alkali-stable side-chain protecting groups and the link to the support (Atherton, E. and Sheppard, R.C. Solid Phase Peptide Synthesis: A Practical Approach IRL Press, New York, N.Y.; 1989).

Alternatively, a linker or spacer molecule is first attached to the free amino groups of the chitosan support. Suitable linkers are linear or branched molecules having at least two functional groups. The linker has a first functional group for coupling to a free amino group of the chitosan and a second functional group for coupling to the carboxyl-terminal of the amino acid of the peptide antigen. An example of a suitable linker is  $\epsilon$ -aminocaproic acid.

The linker may also be a cleavable linker, such as benzhydrylamine (Bernatowicz et al Tetrahedron Letters 30: 4645; 1989) which yields a peptide amide after cleavage or 4-(4-hydroxymethyl-3-methoxyphenyl)-butyric acid (Florsheimer and Riniker, European Peptide Symposium, Barcelona; 1990) which yields a peptide with a carboxy-



terminus after cleavage. The linker is cleaved after peptide synthesis to release the peptide from the chitosan support. The synthesis of a peptide-chitosan complex using a cleavable linker is useful in determining the quality of a peptide-chitosan complex synthesized with a non-cleavable linker (as will be shown in Example 1). The cleaved peptide may also be used for any desired application.

The linker serves to distance the first amino acid from the chitosan support to alleviate steric interference. Furthermore, by providing a linker molecule having a third functional group, immunostimulatory structures can also be introduced by using the third functional group as the point of attachment. Thus, the potential exists for building an adjuvant property into the peptide-chitosan complex.

After addition of the first amino acid or linker, any remaining free amino groups on the chitosan are then acylated with a  $C_2$ - $C_{20}$  fatty acid derivative, for example with acetyl or palmityl groups. This provides a further means for altering the biological properties of the resultant structure.

In the case wherein a linker is first attached to the chitosan support, the carboxyl-terminus of the first Fmoc-amino acid of the desired peptide sequence is then coupled to the free amino groups of the linker.

The Fmoc group of the first amino acid, coupled either directly to the chitosan or via a linker molecule, is then released from the  $\alpha$ -amino group of the first amino acid in a manner known to those skilled in the art, for example, by addition of a base. Stepwise synthesis of the peptide then continues by coupling of additional Fmoc-amino acids and subsequent releasing of the Fmoc group to expose the  $\alpha$ -amino groups for coupling of the next desired amino acid. The synthesis may be conducted in an automatic continuous flow peptide synthesizer, such as, for example, the Milligen 9050 Plus<sup>TM</sup> (Millipore Corp., Milford, Massachusetts, U.S.A.).

Once the desired peptide has been synthesized, the amino acid side-chain protecting groups are removed in a manner known to those skilled in the art, for example, by treatment with 95% trifluoroacetic acid (TFA)/water. The structure is then washed free of unreacted chemicals and the peptide-chitosan complex is used successfully as a peptide antigen presenter with no further purification required.

The resultant peptide-chitosan complex is insoluble at neutral pH. Accordingly, after completion of the synthesis, the chitosan can be fragmented to soluble oligomers using chitinase or chitosanase or by carrying out a partial hydrolysis with anhydrous hydrofluoric acid. Alternatively, the peptide-chitosan complex may be fragmented, using chitinase or chitosanase, prior to removal of the protecting groups from the amino acids.

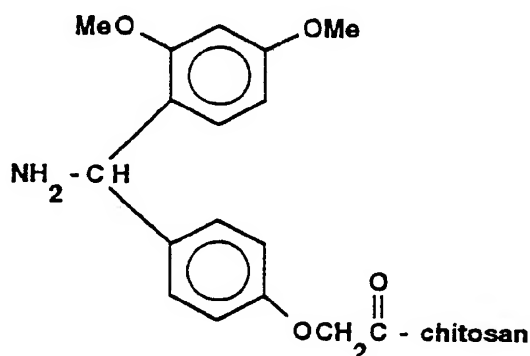
Fragmenting the fully protected peptide-chitosan complex provides the opportunity to derivatize the peptide-chitosan complex fragments. For example, the fragments may be derivatized with acetic anhydride and dimethylamino pyridine in dimethylformamide (DMF) to produce esters of the sugar hydroxyl groups. Any fatty acid anhydride can be used in place of the acetic anhydride, including for example palmityl anhydride. The fragments may also be derivatized by the formation of O-ether derivatives of the sugar hydroxyl groups, for example, by the treatment of the peptide-chitosan complex with methyl iodide plus silver oxide in DMF. In both cases, the Fmoc and side-chain protecting groups on the peptide are finally removed by treatment with organic base followed by 95% TFA in DCM. The fragments have adjuvant properties which increase the immunogenicity of the peptide sequence.

The following Examples illustrate the present invention. Two sequences from human parathyroid hormone (hPTH) were synthesized on chitosan. The use of linkers and cleavable linkers is also illustrated.

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Example 1

Chitosan, derived from squid pen chitin (Sea Fisheries Institute, Gdynia, Poland), was dissolved in 1 M acetic acid and freeze-dried to give a white soft material. The chitosan was about 79% deacetylated, as determined according to the method of Neugebauer et al (Carbohydr Res 189: 363-367; 1989).

A cleavable linker was conjugated to the free amino groups of 150 mg of the chitosan preparation. The cleavable linker was Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine trialkoxybenzhydrylamine (Bernatowicz et al Tetrahedron Letters 30: 4645; 1989). The cleavable linker-chitosan complex had the following formula:



The cleavable linker-chitosan complex and 150 mg of unconjugated chitosan preparation were placed in a column of a continuous-flow peptide synthesizer (Milligen 9050 Plus™). The two types of chitosan were physically separated in the column by a frit. The packed column was washed with dichloromethane (DCM), 20% diisopropylethylamine (DIPEA) in DCM, and DMF. Synthesis of the following peptide sequence was carried out automatically:

**Val-Arg-Ala-Tyr-Asn-Gln-Pro-Ala-Gly-Asp-Val-Arg**

The side-chains of the amino acids were protected in the following manner: (1) the guanido group of arginine was protected as the Pmc (2,2,5,7,8-pentamethyl-chroman-6-

sulfonyl) derivative thereof; (2) the carboxyl group of aspartic acid was protected as the t-butyl ester thereof; (3) the amide nitrogens of glutamine and asparagine were protected as the trityl derivatives thereof; and (4) the hydroxyl group of tyrosine was protected as the t-butyl ester thereof. Amino acid derivatives were purchased from Bachem Chemicals, California, U.S.A. The  $\alpha$ -amino groups of the amino acids were protected with an Fmoc group during coupling.

Couplings were performed by preactivation of the protected amino acids in situ with a mixture of TBTU/HOBT/NMM in DMF. A four-fold excess of activated amino acids was used with double coupling throughout the synthesis. After each coupling step, unreacted amino acids were "capped" by reaction with acetic anhydride in the presence of HOBT to prevent the formation of deletion analogues, which would be formed if the following amino acid residue were added without the capping step and the previous coupling had not gone to completion. The coupling times for arginine and glycine additions were increased from 30 minutes to 60 minutes.

Fmoc deprotection was accomplished by a 6 minute flow at 6 ml/min of 20% piperidine in DMF. The release was monitored at 300 nm.

After attachment of the first amino acid residue, the chitosan was acetylated with acetic anhydride to acetylate any unreacted amino groups on the chitosan.

After completion of the synthesis, the peptide-chitosan complexes were washed, in the column, with DMF, DCM and DMF. Both the cleavable linker-chitosan complex and the peptide-chitosan complex were treated with 95% TFA/water plus 3% each of phenol and thioanisole and 1.5% ethanedithiol ("scavengers") in DCM to cleave the cleavable linker and to remove the side-chain protecting groups. The peptide product was precipitated by dropping into a large excess of diethylether and the precipitate was collected by centrifugation and freeze-dried.

The product was analyzed by HPLC on a Supelco<sup>TM</sup> C<sub>18</sub> silica column (4.6 x 25 mm, 5 µm particle size). The column was eluted with a 0 - 50% gradient of 0.1% TFA/acetonitrile in 0.1% TFA/water. The elution pattern of the crude product, monitored at 214 nm, is shown in Figure 1. The correct product is indicated with an asterisk and was identified by FABMS (Fast Atomic Bombardment Mass Spectroscopy) as having a mass of 1344.3 (expected mass: 1344.55). The peptide amide product had the following sequence:

**Val-Arg-Ala-Tyr-Asn-Gln-Pro-Ala-Gly-Asp-Val-Arg-NH<sub>2</sub>**

The remaining peptide-chitosan complex is suitable for injection into an animal for the generation of an immune response.

#### Example 2

Chitosan, derived from squid pen chitin (Sea Fisheries Institute, Gdynia, Poland), was dissolved in 1 M acetic acid and freeze-dried to give a white soft material. The chitosan was about 79% deacetylated, as determined according to the method of Neugebauer et al (Carbohydr Res 189: 363-367; 1989).

150 mg of the chitosan preparation were placed in a column of a continuous-flow peptide synthesizer (Milligen 9050 Plus<sup>TM</sup>). The packed column was washed with DCM, 20% DIPEA in DCM, and DMF. Synthesis of hPTH-(44-68), having the following sequence:

**Arg-Asp-Ala-Gly-Ser-Gln-Arg-Pro-  
Arg-Lys-Lys-Glu-Asp-Asn-Val-Leu-  
Val-Glu-Ser-His-Glu-Lys-Ser-Leu-Gly**

was carried out automatically.

The side-chains were protected using amino acid derivatives, including the Mtr derivative of arginine; the t-butyl ester of aspartic acid, glutamic acid and serine; the trityl derivative of asparagine, glutamine and histidine; and the t-butyloxycarbonyl derivative of lysine. The  $\alpha$ -amino group of the amino acids were protected by Fmoc groups.

Couplings were performed by preactivation of the protected amino acids in situ with a mixture of 0.3 M TBTU/HOBT in DMF and 0.6 M DIPEA in DMF.

A four-fold excess of activated amino acids was used, based on the total amount of free amino groups theoretically available upon initiation of the synthesis. Double couplings were used for isoleucine and valine, with a preceding solvent exchange into DCM/DMF (1:1 v/v). Fmoc deprotection was accomplished by a 6 minute flow at 6 ml/min of 20% piperidine in DMF. The release of the Fmoc group was monitored at 300 nm.

The peptide was synthesized directly on the free amino groups of the chitosan. After attachment of the first amino acid residue, the chitosan was acylated with palmityl anhydride followed by acetyl anhydride, in 50% pyridine/DCM overnight. After completion of the synthesis, side-chain deprotection, without cleavage from the support, was accomplished with 10 ml of 95% TFA/water in the presence of appropriate scavengers (thioanisole, phenol, and 1,2-ethanedithiol) by stirring for 2 hours to dissolution. Under these conditions, cleavage of the glycosidic bond of the chitosan is expected to be minimal (Otvos et al, Tetrahedron Letters 31: 5889-5892; 1990). The product was precipitated from the deprotection solution with 100 ml of diethyl ether. The final precipitated product was filtered, washed with diethyl ether, suspended in 20% acetic acid, and lyophilized.

Amino acid analysis indicated that about 2% of the hPTH-(44-68)-chitosan complex consisted of peptide. The

hPTH-(44-68)-chitosan complex was washed extensively prior to injection into rabbits (Example 3).

5

**Example 3**

Four New Zealand white rabbits were each initially injected subcutaneously with 0.25 mg of a suspension of the hPTH-(44-68)-chitosan complex of Example 2 in 10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride (phosphate buffered saline, PBS). Three of the rabbits were co-injected with Freund's incomplete adjuvant (FIA). Booster injections, at 6 and 8 weeks after initial injection, contained 2 mg of the complex in 1 ml of PBS so that the total peptide in each injection of the hPTH-(44-68)-chitosan complex was from about 20 to 40  $\mu$ g.

Antibody responses were measured by enzyme-linked immunosorbent assays (ELISA). ELISA assays were performed using hPTH prepared as described in Sung et al (J Biol Chem 266: 2831-2835; 1991). Titer plates were coated with hPTH by incubating each well for 16 hours at 4°C with 100  $\mu$ l of 5  $\mu$ g/ml hPTH in 50 mM sodium carbonate, pH 9.6. After washing three times with 0.02 M Tris, 0.15 M sodium chloride, pH 7.4 (TBS), the wells were saturated by further incubation (1 hour, 22°C) with 1% skim milk powder in 0.02 M Tris, pH 7.4. The plate was then washed three times with TBS, 0.01 M ethylenediamine tetraacetate (EDTA), and 100  $\mu$ l of antisera to the hPTH-(44-68)-chitosan complex diluted into TBS was added to each well.

After incubation at 22°C for 2 hours, the wells were washed three times with TBS, and incubated with 100  $\mu$ l of a 1:500 dilution of alkaline phosphatase conjugated affinity-purified goat anti-rabbit IgG in TBS (2 hours, 22°C). The wells were then washed three times with 1% skim milk powder in TBS, once with diethanolamine buffer (10 mM diethanolamine, pH 9.8, 5 mM  $MgCl_2$ ), and 100  $\mu$ l of 1 mg/ml p-nitrophenylphosphate in ethanolamine buffer was added.

After incubation for 30 minutes at 22°C, the reaction was terminated by the addition of 50  $\mu$ l of 4 M NaOH and the plate was read at 405 nm. A control consisting of an equivalent dilution of pre-immune antisera was subtracted from each reading.

The results of the ELISA assays are shown in Figure 2. Three of the four rabbits were injected with both the hPTH-(44-68)-chitosan complex and FIA. The results are represented by the o, • and  $\Delta$  curves. The fourth rabbit was injected without added FIA ( $\Delta$  curve). Antisera were tested nine weeks after initial injection, i.e. one week after the final injection.

#### Example 4

Chitosan was prepared as described in Example 2. 150 mg of the chitosan preparation were placed in a column of a continuous-flow peptide synthesizer (Milligen 9050 Plus<sup>TM</sup>). The packed column was washed with DCM, 20% DIPEA in DCM, and DMF. A linker residue (N-Fmoc- $\epsilon$ -aminocaproic acid) was coupled 3 times, each time using a four-fold molar excess of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)/DIPEA (1:2.5) to the free amino groups of the chitosan. After washing with DMF, the unreacted amino groups of the chitosan were palmitylated with palmityl anhydride in dry pyridine (1:1 v/v) followed by acetylation in the same manner.

Synthesis of hPTH-(1-17), having the following sequence:

**Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-  
His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser**

was carried out automatically, as described in Example 2. In the synthesis of the hPTH-(1-17) analogue, N-methylmorpholine was used in place of DIPEA.



**Example 5**

The hPTH-(1-17)-chitosan complex of Example 4 was tested as an antigen in rabbits, as described in Example 3.

5 The results of the ELISA assays are shown in Figure 3. No adjuvant was used in several tests of the palmitylated derivatives, and the results were observed to be similar to that obtained in the presence of added Freund's incomplete adjuvant to the injected material.

10 Figure 3 illustrates the ELISA results of antisera from two rabbits injected with the hPTH-(1-17)-chitosan complex with no added FIA.

## WE CLAIM:

1. A process for peptide synthesis on chitosan, comprising the steps of:
  - 5 providing amino acids having side-chain reactive groups thereof blocked with blocking groups;
  - protecting the  $\alpha$ -amino group of the amino acids with a temporary protecting group;
  - attaching a first amino acid to the free amino
  - 10 groups of the chitosan;
  - releasing the temporary protecting group from the attached amino group;
  - sequentially attaching the desired amino acids to the previously attached amino acid and releasing the
  - 15 temporary protecting group therefrom until the desired peptide is synthesized to form a peptide-chitosan complex; and
  - removing the blocking groups from the side-chain reactive groups of the amino acids.
- 20 2. A process for peptide synthesis according to claim 1, further comprising the step of attaching a bifunctional linker molecule to the free amino groups of the chitosan, prior to the step of attaching the first amino acid.
- 25 3. A process for peptide synthesis according to claim 2, wherein the linker molecule is a cleavable linker molecule.
- 30 4. A process for peptide synthesis according to claim 2, wherein the linker molecule has a third functional group.
5. A process for peptide synthesis according to claim 4, further comprising the step of attaching an
- 35 immunostimulatory structure to the third functional group of the linker molecule.

6. A process for peptide synthesis according to claim 1, further comprising the step of capping any remaining free amino groups on the chitosan, after the first amino acid has been attached to the chitosan.

5

7. A process for peptide synthesis according to claim 2, further comprising the step of capping any remaining free amino groups on the chitosan, after the linker molecule has been attached to the chitosan.

10

8. A process for peptide synthesis according to claim 1, further comprising the step of fragmenting the peptide-chitosan complex, after removing the blocking groups therefrom.

15

9. A process for peptide synthesis according to claim 1, further comprising the step of fragmenting the peptide-chitosan complex, prior to removing the blocking groups therefrom.

20

10. A process for peptide synthesis according to claim 9, further comprising the step of derivatizing the fragmented peptide-chitosan complex.

25

11. A peptide-chitosan complex prepared by the process comprising the steps of:

providing a chitosan support;

providing amino acids having side-chain reactive groups thereof blocked with blocking groups;

30

protecting the  $\alpha$ -amino group of the amino acids with a temporary protecting group;

attaching a first amino acid to the free amino groups of the chitosan;

releasing the temporary protecting group from the attached amino group;

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sequentially attaching the desired amino acids to the previously attached amino acid and releasing the

temporary protecting group therefrom until the desired peptide is synthesized to form a peptide-chitosan complex; and

5 removing the blocking groups from the side-chain reactive groups of the amino acids.

12. A peptide-chitosan complex according to claim 11, further comprising a bifunctional linker molecule which is attached to the free amino groups of the chitosan, prior to  
10 the step of attaching the first amino acid.

13. A peptide-chitosan complex according to claim 12, wherein the linker molecule has a third functional group.

15 14. A peptide-chitosan complex according to claim 13, further comprising an immunostimulatory structure attached to the third functional group of the linker molecule.

20 15. A peptide-chitosan complex according to claim 11, wherein any remaining free amino groups have a capping group attached thereto.

16. A peptide-chitosan complex according to claim 11, wherein the peptide-chitosan complex is fragmented.  
25

17. A peptide-chitosan complex according to claim 16, wherein the fragmented peptide-chitosan complex is derivatized.

30 18. A peptide prepared by the process comprising the steps of:

providing a chitosan support;  
providing amino acids having side-chain reactive groups thereof blocked with blocking groups;  
35 protecting the  $\alpha$ -amino group of the amino acids with a temporary protecting group;

attaching a bifunctional cleavable linker molecule to the free amino groups of the chitosan;

attaching a first amino acid to the free amino group of the cleavable linker molecule;

5 releasing the temporary protecting group from the attached amino group;

sequentially attaching the desired amino acids to the previously attached amino acid and releasing the temporary protecting group therefrom until the desired peptide is synthesized to form a peptide-chitosan complex;

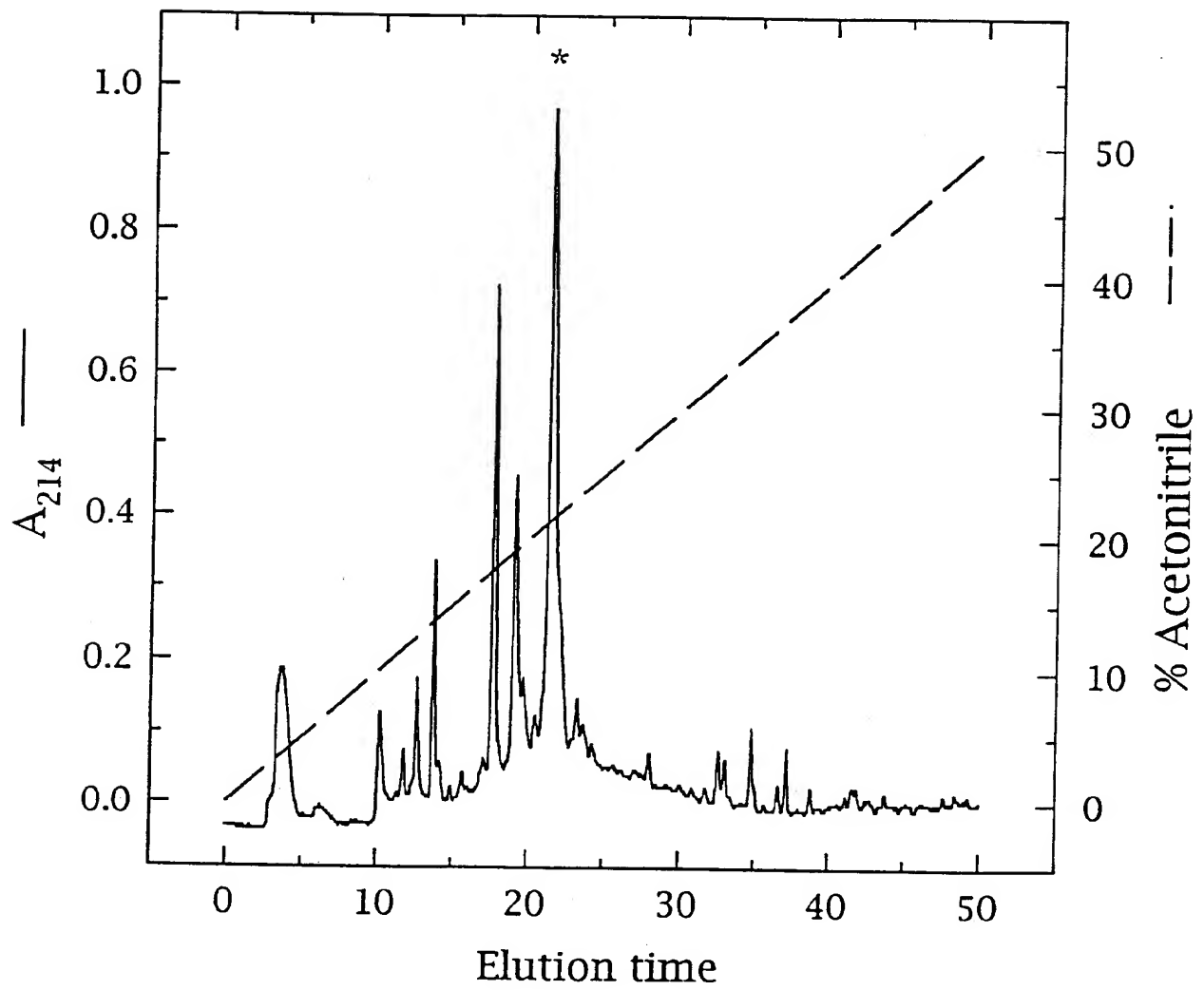
10 cleaving the cleavable linker molecule to release the peptide from the chitosan; and

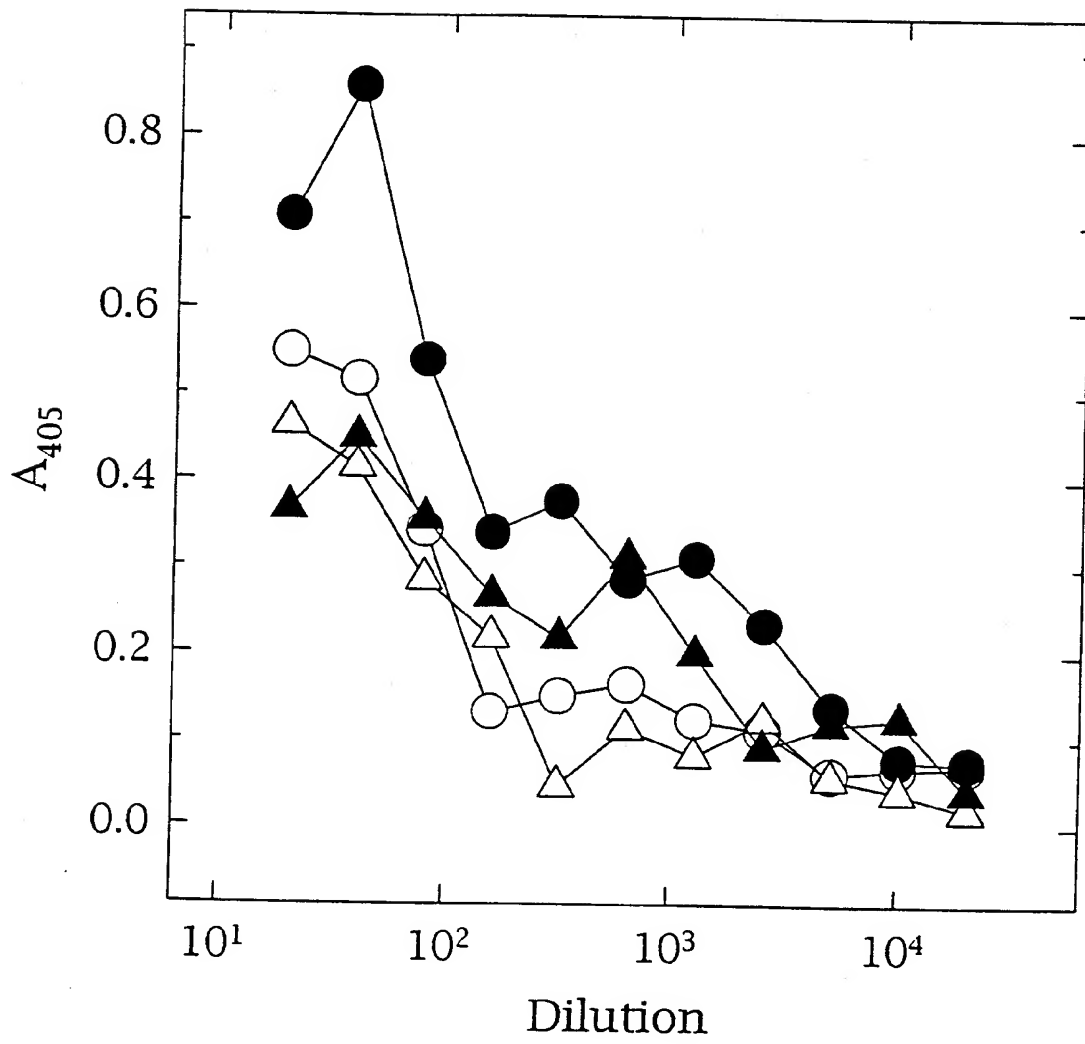
removing the blocking groups from the side-chain reactive groups of the amino acids.

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**FIGURE 1**



**FIGURE 2**

**FIGURE 3**